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Original Article

# Serum levels of miR-199a-5p correlates with blood pressure in premature cardiovascular disease patients homozygous for the *MTHFR* 677C > T polymorphism

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ABSTRACT

This investigation profiled circulating serum concentrations of microRNAs (miRNAs) in premature cardiovascular disease (CVD) patients screened for the 677C > T polymorphism in methylenetetrahydrofolate reductase (MTHFR), a risk factor for hypertension. Serum samples from 75 premature CVD patients of known *MTHFR* genotype were analysed for CVD-related miRNA expression, to identify those that were associated with blood pressure. Samples were collected at baseline and following intervention with riboflavin as part of a randomized controlled trial. In patients with the *MTHFR* 677TT genotype, expression of miR-199a-5p in serum was inversely correlated with hypertension at baseline, and with change in blood pressure in TT genotype patients who responded to riboflavin intervention. These correlations were not observed in *MTHFR* 677CC genotype patients. *In vitro* experiments and *in silico* data analysis provided evidence that miR-199a-5p targets SMAD4. This is the first study to link miR-199a-5p expression with hypertension in a genetically at-risk cohort of premature CVD patients.

## 1. Introduction

Cardiovascular diseases (CVD) are among the leading causes of morbidity and mortality globally [1]. At present elevated blood pressure is the most important predictor of vascular mortality [2], but the underlying causes of hypertension remain unclear. It is becoming increasingly apparent that genetic traits influence the biological processes causing hypertension, leading to the suggestion that specific genetic markers may offer a novel diagnostic approach to inform therapeutic interventions to reduce CVD incidence [3,4]. A new class of genes called microRNAs (miRNAs) have generated much excitement in this respect and show great potential for early diagnosis and tailored treatment for CVD [4–7].

miRNAs are small, non-coding RNA molecules that regulate gene expression by interacting with messenger RNAs (mRNAs). Preliminary studies provide some evidence that the abnormal expression of miRNAs is associated with various degenerative disease states, including CVD [4–6]. Both animal and human studies have identified circulating serum concentrations of different miRNAs as potentially valuable markers of acute myocardial infarction [7], heart failure [8,9], cardiac hypertrophy [10], stroke [11,12], and hypertension [9,13]. However, the reported findings are often based on relatively small samples, with contradictory results reported between studies; thus it is still not certain which miRNAs are the most reliable biomarkers, or which ones exert most influence in CVD development. It is clear that more focused studies are required in order to identify miRNAs which play an important role in CVD progression and to understand how they function. To address this need, we considered how variation in the gene encoding the folate-metabolizing enzyme methylenetetrahydrofolate reductase (MTHFR) may contribute to hypertension by influencing the epigenetic regulation and subsequent expression of miRNAs.

The 677C > T polymorphism in the *MTHFR* gene encoding for MTHFR is associated with an increased risk of CVD, particularly stroke [14–16]. More recently this genetic variant has emerged as an

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independent risk factor for hypertension, with meta-analyses estimating an increased risk of between 24 and 87%, specifically in adults who are homozygous for the polymorphism (*MTHFR* 677TT genotype) [17]. Importantly, MTHFR activity is dependent on an adequate supply of the B-vitamin riboflavin in its co-factor form FAD. Previous work conducted at this centre has demonstrated in 3 separate randomized controlled trials (RCTs) that intervention with riboflavin at the dietary level of 1.6 mg/day can significantly decrease blood pressure in TT hypertensive adults compared with controls [18–20]; no such decrease in blood pressure was observed in the CT or CC genotypes compared with controls [18]. The mean lowering of blood pressure observed across the three studies ranged from 5 to 13 mmHg [17] and although the mechanism of action remains to be established, it has been suggested that epigenetic modification may be implicated [21]. This modification could lead to aberrant expression of important genes, including miRNAs.

No previous study has considered how miRNA expression might be influenced by *MTHFR* genotype. Therefore, this study was intended as a proof-of-principle pilot study to address this gap in the knowledge. The aim was to investigate if miRNA expression differs with *MTHFR* genotype and can be modified in patients whose blood pressure had responded significantly to riboflavin treatment, by analyzing samples from our previously conducted RCTs [18–20]. In doing so, we aimed to explore how miRNAs might be influenced by changes in blood pressure, which would in turn provide insights to the underlying pathogenesis of CVD.

## 2. Materials and methods

### 2.1. Clinical serum samples

The set of serum samples analysed in this study had been collected from premature CVD patients with known *MTHFR* genotype, as part of a previous larger study at Ulster University [18]. Patients were recruited from Altnagelvin Area Hospital, Western Health and Social Services Trust, Northern Ireland, and screened for the 677C > T polymorphism in *MTHFR* (rs1801133) by DNA analysis of a buccal swab sample. CVD was identified by a previous myocardial infarction diagnosed on the basis of ECG changes or angina diagnosed by an exercise stress test. Recruited patients were subsequently randomized to treatment as part of a placebo-controlled double-blind, randomized controlled riboflavin trial. As previously described [18], blood pressure measurements were recorded and non-fasting blood samples were collected at baseline and post-intervention by the same researcher blinded to the genotype and treatment status of the patient. At each time point, two separate measurements were taken 15 min apart, with the patient at rest (10–15 min) in the sitting position, using an Omron 705CP electronic blood pressure monitor (Medisave, Dorset, UK). Weight (kg), height (m), BMI (weight (kg)/height$^2$ (m)) and waist circumference (cm) were also recorded. Plasma total homocysteine and red cell folate pre- and post-intervention were subsequently measured. Similarly, riboflavin status was determined by erythrocyte glutathione reductase activity coefficient (EGRac). Ethical approval was granted by both the Research Ethical Committee at Ulster University and Altnagelvin Area Hospital. All patients completed a health and medical/lifestyle questionnaire and provided informed consent for their tissues to be used in subsequent studies. Use of patient material and information, as well as research protocols, were approved by ORECNNI (Ref. No. 12/NI/0136). For the current study, all available TT samples from the original cohort were used (45 out of 49). 30 matched samples from CC genotype patients were selected for comparison.

### 2.2. microRNA expression profiling

The FirePlex miRNA Assay (Abcam, Cambridge, UK) was used to profile miRNAs in each serum sample. The Cardiology Fixed Panel contained 68 miRNAs that have been previously reported to be differentially regulated in normal or abnormal cardiovascular health (Supplementary Table 1). The panel included general markers for multiple cardiovascular-related diseases, markers specific to specific cardiovascular states or treatment responses, markers that may indicate haemolysis in isolated plasma/serum/exosomes and internal controls to validate assay performance. Results were obtained as flow cytometric fluorescent readouts which were normalized as part of the data analysis.

**Table 1**
Baseline characteristics of premature CVD patients ($n$ = 75) prescreened for the *MTHFR* 677C > T genotype.

| | *MTHFR* 677C > T Genotype | |
|---|---|---|
| | CC ($n$ = 30) | TT (n = 45) |
| Age now (years) | 53.1 | 54.4 |
| Age at event (years) | 47.3 | 47.4 |
| Male (%) | 76.7 | 82.2 |
| Family History of CVD (%) | 73.3 | 68.9 |
| Current Smoker (%) | 30.8 | 33.3 |
| BMI (kg/m$^2$) | 28.4 | 29.2 |
| Waist Circumference (cm) | 96.8 | 94.3 |
| Blood Pressure | | |
| Systolic Blood Pressure (mmHg) | 117.9 | 144.3*** |
| Diastolic Blood Pressure (mmHg) | 74.8 | 86.9*** |

Data are expressed as the mean unless otherwise indicated. Statistical significance was assessed by one-way ANOVA with Tukey posthoc test (*P*-values: *$p$ < .05, **$p$ < .01, ***$p$ < .001).

### 2.3. Cell culture & transfections

Human Umbilical Vein Endothelial Cells (HUVEC) cell-line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in ATCC-formulated F-12 K (Kaighn's Modification of Ham's F-12 with L-glutamine) growth medium (ATCC) supplemented with 0.1 mg/ml heparin and 0.03 mg/ml endothelial cell growth supplement (ECGS) (both Sigma-Aldrich) and adjusted to a final concentration of 10% Fetal Bovine Serum (Life Technologies). Prostate epithelial cell-line RWPE1 was cultured in keratinocyte growth medium supplemented with 5 ng/ml human recombinant epidermal growth factor and 0.05 mg/ml bovine pituitary extract (Life Technologies, Paisley, UK). All cells were grown on gelatine (Sigma-Aldrich) coated tissue culture flasks and plates in an incubator with a humidified atmosphere of 95% air and 5% $CO_2$ at 37 °C and routinely passaged. For miRNA transfection, cells were seeded at 100,000 cells/well in a 6-well plate. After 24 h, cells were transfected with miR-199a (pre-miR-199a-5p) or non-targeting negative control (pre-neg) (both Life Technologies) at a final concentration of 25 nM using Lipofectamine 2000 (Life Technologies). After 72 h, cells were harvested for RNA extraction.

### 2.4. PCR analysis

RNA extraction was carried out using Trizol (Life Technologies) according to manufacturer's instructions. For gene analysis, 1 µg RNA was used for first strand cDNA synthesis using random primers with transcriptor high-fidelity cDNA synthesis kit (Roche, Sussex, UK) according to manufacturer's instructions. For quantitative real-time PCR (qRT-PCR), amplification of PCR products was quantified using FastStart SYBR Green Master (Roche) on a Roche LC480 Lightcycler, using primer sets for *SMAD4* (Forward 5'-AAGGTTCCTTCAAGCTG CCC-3′; Reverse 5'-CAATGGCTTCTGTCCTGTGGA-3′), *VEGFα* (Forward 5'-AGCTACTGCCATCCAATCGA-3′; Reverse 5'-GGTGAGGTTTGATCCG CATA-3′), and housekeeping gene *β-actin* (Forward 5'-GGACTTCGAGC AAGAGATGG-3′; Reverse 5'-AGCACTGTGTTGGCGTACAG-3′). Expression was normalized to *β-actin* and graphs represent the

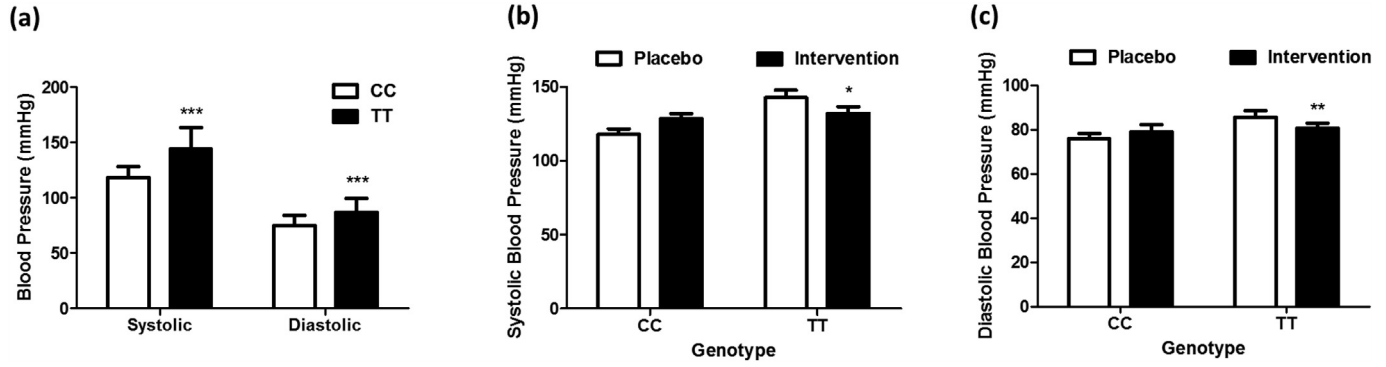


**Fig.. 1.** Blood Pressure in Premature CVD Patients Pre- and Post- Intervention with Riboflavin.
(a) In pre-intervention samples, patients with the TT genotype have a significantly higher systolic and diastolic blood pressure at compared to the CC genotype. Following riboflavin intervention (1.6 mg per day, 16 weeks), patients with the TT genotype had a significant decrease in both (b) systolic and (c) diastolic blood pressure in comparison to patients with the CC genotype. These findings were in keeping with those of the original study [18]. Statistical significance was assessed by paired two tailed *t*-test. (*P*-values: **p* < .05, **p < .01, ****p* < .001).

**Table 2**
Blood pressure response to riboflavin intervention (1.6 mg per day, 16 weeks).

| | *MTHFR* 677C > T Genotype | | | |
|---|---|---|---|---|
| | CC (n = 30) | | TT (*n* = 45) | |
| | Placebo | Riboflavin | Placebo | Riboflavin |
| Systolic Blood Pressure (mmHg) | | | | |
| Before | 112.8 | 123 | 146.6 | 142 |
| After | 117.8 | 128.5 | 142.7 | 132 |
| Change | +5 | +5.5 | −3.9 | −10* |
| Diastolic Blood Pressure (mmHg) | | | | |
| Before | 73.1 | 76.5 | 85.7 | 88 |
| After | 76.1 | 78.8 | 85.7 | 81 |
| Change | +2.9 | +2.3 | 0 | −8** |

Data are expressed as the mean unless otherwise indicated. Statistical significance was assessed by paired two tailed t-test. (*P*-values: **p* < .05, ***p* < .01, ****p* < .001).

combined results of three independent biological replicates.

For microRNA analysis, qRT-PCR for miR-199a-5p was performed using the miRCURY LNA™ microRNA PCR system (Exiqon, Vedbaek, Denmark). 20 ng template RNA was used in each first strand cDNA synthesis reaction. PCR was performed over 40 amplification cycles and fluorescence monitored on the Roche LC480 Lightcycler. For all qRT-PCR miRNA analysis, normalization was against U6snRNA and graphs represent the combined results from 3 independent biological replicates, unless otherwise indicated.

### 2.5. Statistical analysis

One-way ANOVA with Tukey posthoc test or unpaired two-tailed Student's *t*-test was used to compare baseline characteristics among the genotype groups. The response to riboflavin intervention was examined by conducting a within–between repeated measures ANOVA for both systolic and diastolic blood pressures (mmHg). The between patient factors were genotype (CC *vs* TT) and intervention group (placebo *vs* riboflavin) with time (before and after) as the within-patient factor. Adjustments for multiple comparisons using Bonferroni were incorporated in the analyses. The FirePlex Analysis Software package (Abcam) was used to analyse miRNA expression. Normalization of all sample data was performed using the geNORM algorithm. The three optimal normalizers identified (let-7i-5p, miR-16-6p and miR-92a-3p) were combined into a single normalization vector which was subsequently applied across the entire dataset. Subsequent analysis of normalized data was performed by Pearson's two-tailed correlation, two-tailed Student's *t*-test and ANOVA with Tukey posthoc test. In all analyses *p*-value thresholds of ****p* < .001, ***p* < .01, **p* < .05 were applied. Data-mining, network mapping, KEGG pathway and Gene Ontology analysis was performed using statistical resources freely available through miRTarbase (http://mirtarbase.mbc.nctu.edu.tw/index.php [22] and the Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resource (http://david.ncifcrf.gov/) [23,24].

## 3. Results

### 3.1. Baseline characteristics of premature CVD patients

The pre-intervention baseline characteristics of participants (*n* = 75) by *MTHFR* genotype, included in the current analysis is shown in Table 1. In this subset of 75 samples, we confirmed that the patients with the TT genotype had a significantly higher systolic (*p* < .001) and diastolic blood pressure (p < .001) at baseline compared to the CC genotype (Fig. 1a), in accordance with findings from the previous study [18].

### 3.2. Blood pressure response to riboflavin intervention

When the samples selected for the current investigation were split into those obtained from patients in the placebo or riboflavin treatment groups, a significant decrease in both systolic (-10 mmHg) and diastolic (-8 mmHg) blood pressure following intervention with riboflavin was observed in patients with TT genotype, whereas no significant reduction was evident in the CC genotype group (Table 2, Fig. 1b and c). The extent of the blood pressure response and the genotype-specific effect of riboflavin were similar to those effects reported previously in the larger cohort [18].

### 3.3. Cluster analysis heat map of miRNA expression

The serum expression of 68 miRNAs was profiled using a cardiology focus panel. Patients were grouped into their respective *MTHFR* genotype and a cluster analysis heat map was used to illustrate the relative expression levels of each miRNA following normalization (Supplementary Fig. 1a). In all samples miRNA expression was successfully measured and profiled. No overall differences were noted between expression of any individual miRNA in the CC and TT genotype groups. However, it was noted that miR-199a-5p had a fold increase of 1.47 in TT genotype samples compared to CC genotype samples, although this fell just short of statistical significance (*p* = .053) (Supplementary Fig. 1b). Therefore, its expression was examined in more detail.

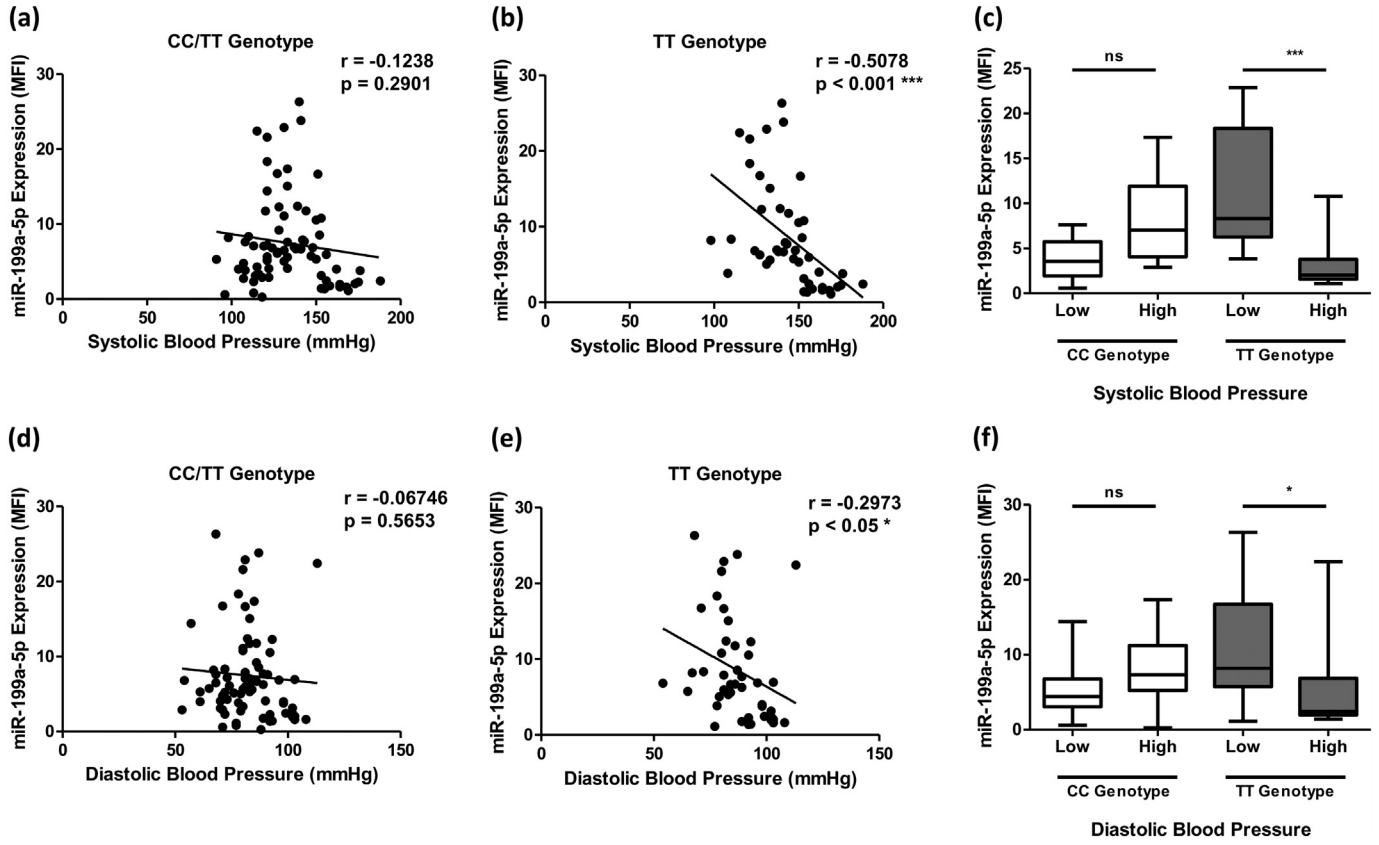


**Fig. 2.** Correlation of miR-199a-5p Expression with Blood Pressure.
(a) A slight trend to inverse correlation was observed for miR-199a-5p with systolic blood pressure in all patients (n = 75) prior to intervention. (b) This inverse correlation became highly significant in patients with TT genotype (n = 45). (c) TT patients in highest tertile of systolic BP ($n$ = 15: mean = 165 ± 10.17 mmHg) showed significantly lower levels of miR-199a-5p than those in the lowest tertile (n = 15: mean = 123 ± 10.93 mmHg). No significant difference in miR-199-5p expression was noted when the same analysis was performed on CC patients in the highest ($n$ = 10: mean = 128.1 ± 4.88 mmHg) and lowest (n = 10: mean = 106.6 ± 7.76 mmHg) tertile of systolic BP. (d) - (f) A similar inverse correlation was observed between miR-199a-5p and diastolic BP. TT patients in highest tertile of diastolic BP (n = 15: mean = 100 ± 5.72 mmHg) showed significantly lower levels of miR-199a-5p than those in the lowest tertile (n = 15: mean = 74 ± 7.85 mmHg). No significant difference in miR-199-5p expression was noted for CC patients in the highest (n = 10: mean = 65 ± 6.50 mmHg) and lowest (n = 10: mean = 84.7 ± 4.00 mmHg) tertile of diastolic BP. Significance was assessed by Pearson's two tailed correlation (A, B, D, E) or one-way ANOVA with Tukey posthoc test (C & F). (P-values: *p < .05, **p < .01, ***p < .001, ns; no significance).

### 3.4. Correlation of miR-199a-5p expression with blood pressure

The expression of miR-199a-5p was of interest as previous reports suggest a link between miR-199a-5p and hypertension [25–27]. In this study a correlation of miR-199a-5p expression with systolic blood pressure in all 75 patients revealed only a slight trend towards an inverse correlation (Fig. 2a). However, when this analysis was confined to patients with the TT genotype, a highly significant inverse correlation was observed (Fig. 2b). Further correlation of miR-199a-5p expression with blood pressure in patients in the highest systolic blood pressure tertile ($n$ = 15; mean = 165 ± 10.17 mmHg) and the lowest tertile (n = 15; mean = 123 ± 10.93 mmHg) was performed. This sub-group analysis revealed that TT genotype patients with higher systolic blood pressure showed significantly lower levels of miR-199a-5p expression than those TT genotype patients with lower systolic blood pressure (Fig. 2c).

A similar inverse correlation was observed in relation to diastolic blood pressure. A slight trend towards an inverse correlation was evident in all patients (Fig. 2d), but this became significant when analysis was confined to the TT genotype patients only (Fig. 2e). Similarly, the TT genotype patients in the highest tertile of diastolic blood pressure ($n$ = 15; mean = 100 ± 5.72 mmHg) showed significantly lower levels of miR-199a-5p expression than those TT genotype patients in the lowest tertile of diastolic blood pressure (n = 15; mean = 74 ± 7.85 mmHg) (Fig. 2f). It is worth noting that in CC genotype patients, a positive correlation was shown between miR-199a-5p expression and systolic blood pressure (Supplementary Fig. 2a). However, no significant correlation was observed for miR-199a-5p expression and diastolic blood pressure (Supplementary Fig. 2b), nor was any significant difference in miR-199a-5p expression noted when samples from patients with highest and lowest blood pressure tertiles in this cohort were compared (Fig. 2c and f).

### 3.5. Correlation of miR-199a-5p Expression with Blood Pressure in Response to Riboflavin Intervention in Patients with TT genotype

Given the inverse correlation observed above, the question remained as to whether the reduction in blood pressure observed in response to riboflavin intervention in the TT genotype patients would be accompanied by increased expression of miR-199a-5p expression in individual patients. To examine this, it was necessary to filter the TT genotype cohort to focus on 'TT responders'. When the change in expression of miR-199a-5p was correlated with the change in systolic blood pressure in the TT genotype cohort as a whole ($n$ = 45), only a trend towards an inverse correlation was revealed (Fig. 3a). Notably, this inverse trend was more evident in TT genotype patients treated with riboflavin ($n$ = 23) (Fig. 3b), whereas no trend towards an inverse correlation was observed at all in TT patients treated with placebo ($n$ = 22) (Fig. 3c). When the riboflavin group were further filtered to analyse only the patients who showed a decrease in systolic blood

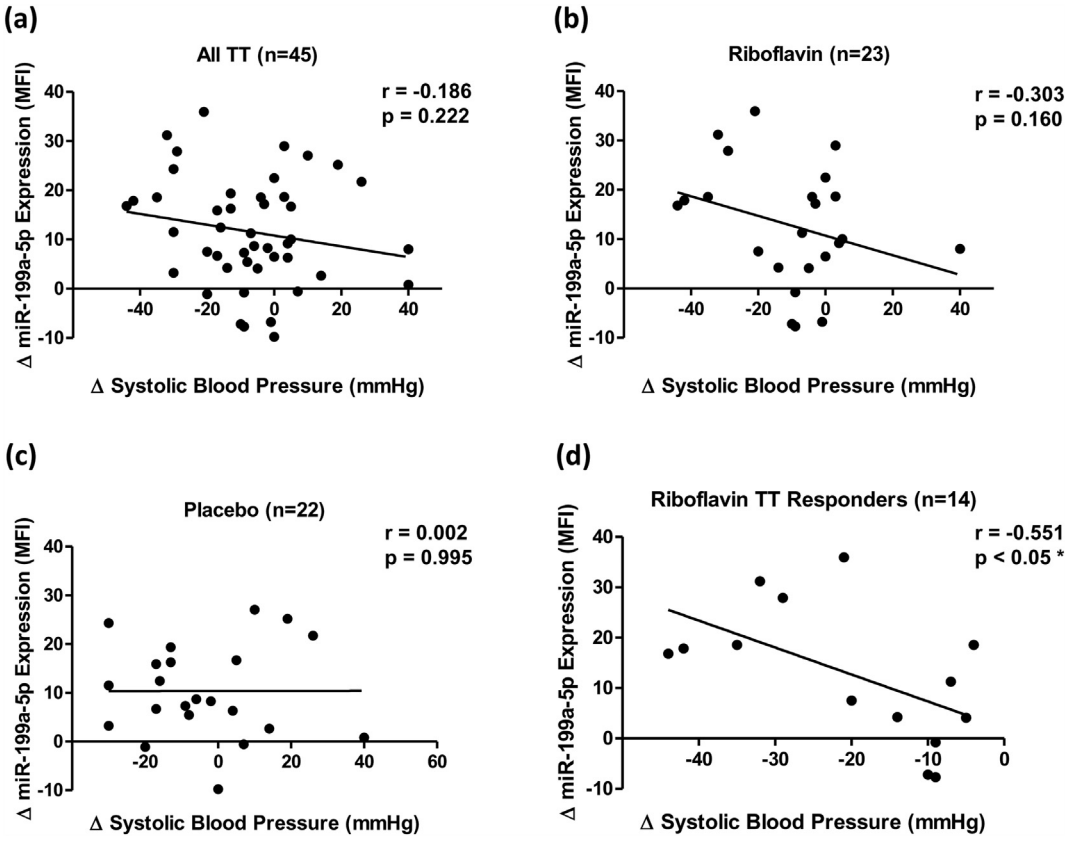


**Fig. 3.** Correlation of miR-199a-5p Expression with Blood Pressure in Response to Riboflavin Intervention.
Analysis of change in miR-199a-5p expression with change in systolic blood pressures in (a) the TT cohort as a whole ($n$ = 45) and (b) those treated with riboflavin (n = 23) revealed a trend towards inverse correlation. (c) No inverse correlation is apparent in placebo treated TT patients ($n$ = 22). (d) However, in patients who responded to riboflavin intervention with a decrease in blood pressure (TT responders; $n$ = 14), a significant inverse correlation was observed. Significance in all graphs was assessed by Pearson's two tailed correlation. (P-value: *p < .05).

pressure in response to riboflavin intervention ($n$ = 14), a significant inverse correlation was noted (Fig. 3d). Linear regression analyses corroborated these results (Supplementary Fig. 3). Thus, in these TT responders, those who responded with the greatest decrease in blood pressure had the greatest increase in miR-199a-5p expression, although the mechanisms underlying these changes are unknown. Therefore, further *in vitro* and *in silico* work was performed to explore what these mechanisms might be.

### 3.6. Role of miR-199a-5p in hypertension

The role of miR-199a-5p in hypertension has not been extensively studied, but several target genes for miR-199a-5p have been identified. To examine how miR-199a-5p might contribute to hypertension, functional annotation analysis of these target genes was performed using the DAVID Bioinformatics Resource [23,24]. Importantly, many miR-199a-5p target genes were revealed to be significantly associated with CVD and hypertension (Supplementary Table 2). Further gene ontology analysis demonstrated a significant association between these targets and cellular processes related to cardiac and vascular development, again illustrating how abnormal expression of miR-199a-5p could influence CVD progression through altered regulation of its target genes (Supplementary Table 3). Likewise, KEGG pathway results revealed a significant enrichment of miR-199a-5p target genes in cellular mechanisms which have been implicated in development of hypertension (Supplementary Table 4) [28–33]. To further investigate novel functionality of this miRNA, its specific impact upon selected target genes was examined (Fig. 4). Interestingly, one predicted target gene of miR-199a-5p was *SMAD4* (Supplementary Table 4, Fig. 4a), which has been previously implicated in hypertension [27,34]. To validate this potential link, miR-199a-5p was over-expressed in HUVEC cells and the effect on *SMAD4* expression measured by PCR. As expected, *SMAD4* expression was significantly reduced when miR-199a-5p levels were increased, which would be expected if *SMAD4* was a true target of miR-199a-5p (Fig. 4b). As a control, expression of *VEGFA*, a more established target of miR-199a-5p [35,36] was examined and was also found to be decreased by miR-199a-5p over-expression. A similar reduction of *SMAD4* expression was observed when we repeated this experiment in RWPE1 epithelial cells (Supplementary Fig. 4a). Further evidence that miR-199a-5p targets *SMAD4* comes from two independent datasets, generated from a study on human aortic valvular endothelial cells [37] and from a cohort of prostate biopsy samples from The Cancer Genome Atlas [38]. In both of these, a significant inverse correlation between miR-199a-5p and *SMAD4* expression is observed (Fig. 4c and Supplementary Fig. 4b). Network analysis of this interaction demonstrated how both miR-199a-5p and *SMAD4* can also influence other genes which contribute to hypertension (Fig. 4d).

## 4. Discussion

In this study we set out to investigate if miRNA expression differs with *MTHFR* genotype and can be modified in patients whose blood pressure had responded significantly to riboflavin treatment, by analyzing samples from our previously conducted RCTs [18–20]. We have successfully demonstrated that we can measure and profile miRNA expression in archived serum samples collected from premature CVD patients. These patients were recruited as part of a previous study which demonstrated that riboflavin supplementation could significantly

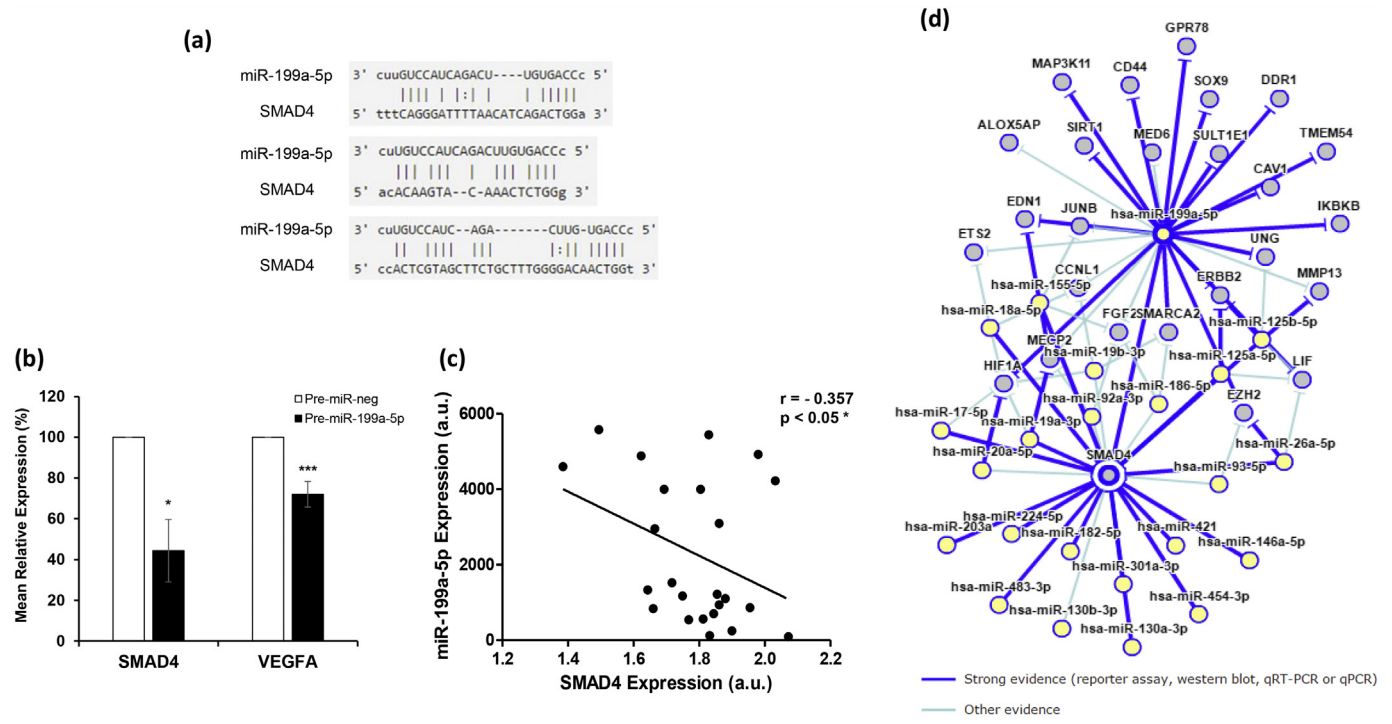


**Fig. 4.** Network Analysis of miR-199a-5p Interactions.
(a) miRanda predicted pairing of three putative target regions in 3' UTR of *SMAD4* and miR-199-5p. (b) qRT-PCR shows that over-expression of miR-199a-5p in HUVEC cells causes *SMAD4* and *VEGFA* levels to be significantly decreased. Significance was assessed by Student two tailed t-test. (P-values: *p < .05, ***p < .001). (c) Inverse correlation between miR-199-5p and *SMAD4* expression in human aortic valvular endothelial cells. Public Dataset ID: GSE26953; array data from Holliday et al. [37]. Significance was assessed by Pearson's two tailed correlation (P-values: *p < .05). (a.u.: arbitrary units) (d) Network analysis using miRTarbase [20] reveals that miR-199a-5p impacts upon *SMAD4* as well as several other targets many of which are known to contribute to hypertension. Likewise, *SMAD4* is regulated by several miRNAs. Accession ID: MIRT007013 [miRNA, hsa-miR-199a-5p:: *SMAD4*, target gene].

lower blood pressure in individuals with the *MTHFR* 677TT genotype [18]. Thus, we had a unique set of archived serum samples, pre and post a highly effective intervention, in which we could test whether any circulating miRNAs correlated with change in blood pressure. From the 68 miRNAs analysed, miR-199-5p emerged as the most promising candidate for further investigation.

As this was a pilot study, we had a relatively small set of samples to work with, all of which were taken from at-risk CVD patients. A wide variation in miRNA expression was noted, meaning no single miRNA was differentially expressed in CC and TT genotype patients at baseline. However, we did note that miR-199a-5p was increased in TT genotype samples compared to CC genotype samples, even though this fell just short of statistical significance; this observation was interesting because miR-199a-5p had previously been linked with hypertension [25]. We therefore proceeded to directly correlate miR-199a-5p serum expression and blood pressure. This analysis showed that expression of miR-199a-5p at baseline was significantly inversely correlated with blood pressure in the serum of *MTHFR* 677TT genotype patients. Moreover, when pre- and post-intervention samples from *MTHFR* 677TT genotype patients were analysed, an increase in miR-199a-5p expression was inversely correlated with a decrease in blood pressure. In CC genotype patients, these correlations were not apparent; only one significant correlation between miR-199a-5p expression and systolic blood pressure was observed. This suggests that the serum expression of miR-199a-5p in *MTHFR* 677TT genotype patients is somehow linked to hypertension only in the TT group of individuals, although the reasons for this require further investigation.

Altered expression of miR-199a-5p is noteworthy as there is some evidence that miR-199a-5p plays a role in CVD. One comprehensive study showed how the hypoxia-inducible cluster miR-199a ~ 214, which includes miR-199a-5p, was functionally linked to hypertension and possible heart failure [25]. Upregulation of miR-199a-5p has been reported in lung tissue from mouse and rat models of pulmonary artery hypertension (PAH), firmly implicating it in the development of this disease [26]. These results were corroborated by an independent study of PAH in animal models, which also identified *SMAD3* as a target of miR-199a-5p [27]. Additionally, the expression of miR-199a-5p was increased in the lung tissues of patients with chronic obstructive pulmonary disease (COPD) [39], and similar results were shown in ventricular hypertrophy diseases [10]. Further evidence of a link with hypoxic stress comes from studies reporting that *HIF-1α* is a target of miR-199a-5p [39,40], and suggests it may be a key regulator in the cellular response to hypoxia. It therefore seems clear that miR-199a-5p plays an important role in mechanisms linked to hypoxic stress and may contribute to hypertension and pathogenesis of various CVD. In this study, we provide further evidence for an important role for miR-199a-5p in mechanisms related to vascular and cardiac processes. We also identify *SMAD4* as a likely target of miR-199a-5p by *in vitro* and *in silico* analysis. This link is important because defects in SMAD-dependent signalling has been linked to hypertension and cardiac pathologies, primarily through their association with TGF-β pathways [27,34]. Therefore, abnormal miR-199a-5p expression will reflect perturbations in these pathways although it remains uncertain whether its altered expression is the cause or result of dysregulated signalling.

Although miR-199a-5p has been previously linked with CVD, only one other study to our knowledge has specifically reported miR-199a-5p as a circulating serum marker of hypertension. A retrospective human study by Hromadnikova et al. (2016) found that down-regulation of miR-199a-5p in maternal peripheral blood was associated with gestational hypertension, preeclampsia, and intrauterine growth restriction [41]. As the most directly comparable study to ours, it is notable that the inverse correlation of miR-199a-5p with blood pressure found in that study agrees with our current findings for patients with *MTHFR* 677TT genotype. Interestingly, the authors also concluded that

dysregulation of miR-199a-5p may be linked to epigenetic changes induced by pregnancy-related complications. This finding has relevance for our current study as MTHFR is a key enzyme in the folate metabolism pathway and therefore influences DNA methylation. Indeed, aberrant hypermethylation and expression of genes involved in DNA methylation has been reported in patients with the variant *MTHFR* 677TT genotype [42–44] and epigenetic mechanisms are known to contribute to hypertension [45,46]. If epigenetic regulation of miR-199a-5p is similarly affected, this may explain why the correlation of miR-199a-5p with blood pressure was only apparent in the TT group of patients. Furthermore, it might explain why changes in miR-199a-5p correlated with blood pressure reduction in the TT patients who responded to riboflavin treatment. Significantly, there is evidence from COPD studies that miR-199a-5p is indeed regulated by methylation status of its promoter [47], so it is tempting to speculate that its expression is affected by aberrant DNA methylation in patients with the *MTHFR* 677TT genotype. Altered expression of miR-199a-5p in TT responders may be owing to riboflavin altering the epigenetic regulation of miR-199a-5p, which in turn may then help reduce blood pressure in this cohort through regulation of genes such as *SMAD4*. Further work in our lab intends to investigate the link between miR-199a-5p and its epigenetic regulation, which may help explain why it displays differential expression in the different disease models discussed above.

In summary, the design of our previous randomized trial which generated the samples for the current analysis provided a unique opportunity to investigate the association between miRNA expression and blood pressure in premature CVD patients with a genetic risk factor for hypertension. Consequently, this is the first study to correlate miR-199a-5p expression with blood pressure in patients with the *MTHFR* 677TT genotype. Furthermore, this study also provides evidence that changes in miR-199a-5p expression are associated with changes in blood pressure in this at-risk group. It is of course important to acknowledge that this study has only investigated a small number of samples, so similar testing of a larger patient cohort would be required to definitively state that miR-199a-5p is a true marker of hypertension in patients with the *MTHFR* 677TT genotype. It would also be useful to include a cohort of patients carrying the polymorphism in heterozygosity (C/T) and perform the same evaluations to further understand the role of miR-199a-5p in hypertension. Nevertheless, even with a relatively small sample set, it is encouraging that our data led us to highlight miR-199a-5p, a miRNA which others have associated with hypertension in independent studies. Furthermore, in identifying *SMAD4* as a probable target, we have also proposed a plausible biological pathway whereby miR-199a-5p could contribute to hypertensive mechanisms. We can therefore speculate that miR-199a-5p plays an important role in the pathogenesis of CVD, particularly in at-risk individuals. Although further investigation is needed to unravel the complex genetic mechanisms involved in the development of essential hypertension, this study demonstrates the value of miRNA serum profiling as an analytical tool to investigate and understand the underlying pathogenesis of CVD.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2019.04.019.

## References

[1] C.M. Lawes, S. Van der Hoorn, A. Rodgers, International Society of Hypertension. Global burden of blood-pressure-related disease, 2001, Lancet 371 (2008) 1513–1518.
[2] S. Lewington, R. Clarke, N. Qizilbash, R. Peto, R. CollinsProspective Studies Collaboration, Age-specific relevance of usual blood pressure to vascular mortality: a meta-analysis of individual data for one million adults in 61 prospective studies, Lancet 360 (2002) 1903–1913.
[3] A. Caroli, M.T. Cardillo, R. Galea, L.M. Biasucci, Potential therapeutic role of microRNAs in ischemic heart disease, J. Cardiol. 61 (2013) 315–320.
[4] A.S. Sayed, K. Xia, U. Salma, T. Yang, J. Peng, Diagnosis, prognosis and therapeutic role of circulating miRNAs in cardiovascular diseases, Heart Lung Circ. 23 (2014) 503–510.
[5] A. Wronska, I. Kurkowska-Jastrzebska, G. Santulli, Application of microRNAs in diagnosis and treatment of cardiovascular disease, Acta Physiol. (Oxf) 213 (2015) 60–83.
[6] S.S. Ali, C. Kala, M. Abid, N. Ahmad, U.S. Sharma, N.A. Khan, Pathological microRNAs in acute cardiovascular diseases and microRNA therapeutics, J. Acute Dis 5 (2016) 9–15.
[7] G.K. Wang, J.Q. Zhu, J.T. Zhang, Q. Li, Y. Li, J. He, et al., Circulating microRNA: a novel potential biomarker for early diagnosis of acute myocardial infarction in humans, Eur. Heart J. 31 (2010) 659–666.
[8] A.J. Tijsen, E.E. Creemers, P.D. Moerland, L.J. de Windt, A.C. van der Wal, et al., MiR423-5p as a circulating biomarker for heart failure, Circ. Res. 106 (2010) 1035–1039.
[9] S.P. Romaine, F.J. Charchar, N.J. Samani, M. Tomaszewski, Circulating microRNAs and hypertension–from new insights into blood pressure regulation to biomarkers of cardiovascular risk, Curr. Opin. Pharmacol. 27 (2016) 1–7.
[10] R. Roncarati, C. Viviani Anselmi, M.A. Losi, L. Papa, E. Cavarretta, P. Da Costa Martins, et al., Circulating miR-29a, among other up-regulated microRNAs, is the only biomarker for both hypertrophy and fibrosis in patients with hypertrophic cardiomyopathy, J. Am. Coll. Cardiol. 63 (2014) 920–927.
[11] P. Wu, X. Zuo, A. Ji, Stroke-induced microRNAs: the potential therapeutic role for stroke, Exp. Ther. Med. 3 (2012) 571–576.
[12] S. Sepramaniam, J.R. Tan, K.S. Tan, D.A. DeSilva, S. Tavintharan, F.P. Woon, et al., Circulating microRNAs as biomarkers of acute stroke, Int. J. Mol. Sci. 15 (2014) 1418–1432.
[13] S. Li, J. Zhu, W. Zhang, Y. Chen, K. Zhang, L.M. Popescu, et al., Signature microRNA expression profile of essential hypertension and its novel link to human cytomegalovirus infection, Circulation 124 (2011) 175–184.
[14] D.S. Wald, M. Law, J.K. Morris, Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis, BMJ 325 (2002) 1202–1206.
[15] M. Klerk, P. Verhoef, R. Clarke, H.J. Blom, F.J. Kok, E.G. Schouten, MTHFR 677C > T polymorphism and risk of coronary heart disease: a meta-analysis, JAMA 288 (2002) 2023–2031.
[16] M.V. Holmes, P. Newcombe, J.A. Hubacek, R. Sofat, S.L. Ricketts, J. Cooper, et al., Effect modification by population dietary folate on the association between MTHFR genotype, homocysteine, and stroke risk: a meta-analysis of genetic studies and randomised trials, Lancet 378 (2011) 584–594.
[17] H. McNulty, J.J. Strain, C.F. Hughes, M. Ward, Riboflavin, MTHFR genotype and blood pressure: a personalized approach to prevention and treatment of hypertension, Mol. Asp. Med. 53 (2017) 2–9.
[18] G. Horigan, H. McNulty, M. Ward, J.J. Strain, J. Purvis, J.M. Scott, Riboflavin lowers blood pressure in cardiovascular disease patients homozygous for the 677C–> T polymorphism in MTHFR, J. Hypertens. 28 (2010) 478–486.
[19] C.P. Wilson, H. McNulty, M. Ward, J.J. Strain, T.G. Trouton, B.A. Hoeft, et al., Blood pressure in treated hypertensive individuals with the MTHFR 677TT genotype is responsive to intervention with riboflavin: findings of a targeted randomized trial, Hypertension 61 (2013) 1302–1308.
[20] C.P. Wilson, M. Ward, H. McNulty, J.J. Strain, T.G. Trouton, G. Horigan, et al., Riboflavin offers a targeted strategy for managing hypertension in patients with the MTHFR 677TT genotype: a 4-y follow-up, Am. J. Clin. Nutr. 95 (2012) 766–772.
[21] A. McMahon, H. McNulty, C.F. Hughes, J.J. Strain, M. Ward, Novel approaches to investigate one-carbon metabolism and related B-vitamins in blood pressure, Nutrients 8 (2016) E720.
[22] C.H. Chou, N.W. Chang, S. Shrestha, S.D. Hsu, Y.L. Lin, W.H. Lee, et al., miRTarBase 2016: updates to the experimentally validated miRNA-target interactions database, Nucleic Acids Res. 44 (D1) (2016) D239–D247.
[23] D.W. Huang, B.T. Sherman, R.A. Lempicki, Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources, Nat. Protocol. 4 (2009) 44–57.
[24] D.W. Huang, B.T. Sherman, R.A. Lempicki, Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists, Nucleic Acids Res. 37 (2009) 1–13.
[25] H. el Azzouzi, S. Leptidis, E. Dirkx, J. Hoeks, B. van Bree, K. Brand, et al., The hypoxia-inducible microRNA cluster miR-199a approximately 214 targets myocardial PPARdelta and impairs mitochondrial fatty acid oxidation, Cell Metab. 18 (2013) 341–354.
[26] H.C. Stevens, L. Deng, J.S. Grant, K. Pinel, M. Thomas, N.W. Morrell, et al., Regulation and function of miR-214 in pulmonary arterial hypertension, Pulm. Circ. 6 (2016) 109–117.
[27] Y. Liu, G. Liu, H. Zhang, J. Wang, MiRNA-199a-5p influences pulmonary artery hypertension via downregulating Smad3, Biochem. Biophys. Res. Commun. 473 (2016) 859–866.
[28] E. Rojas, D. Rodríguez-Molina, P. Bolli, Z.H. Israili, J. Faría, E. Fidilio, et al., The role of adiponectin in endothelial dysfunction and hypertension, Curr. Hypertens. Rep. 16 (2014) 463.
[29] D.O. dos Santos, V. Blefari, F.P. Prado, C.A. Silva, R. Fazan Jr., H.C. Salgado, et al., Reduced expression of adherens and gap junction proteins can have a fundamental

role in the development of heart failure following cardiac hypertrophy in rats, Exp. Mol. Pathol. 100 (2016) 167–176.
[30] R.M. Tuder, S.L. Archer, P. Dorfmüller, S.C. Erzurum, C. Guignabert, E. Michelakis, et al., Relevant issues in the pathology and pathobiology of pulmonary hypertension, J. Am. Coll. Cardiol. 62 (2013) D4–12.
[31] N. Queisser, N. Schupp, Aldosterone, oxidative stress, and NF-κB activation in hypertension-related cardiovascular and renal diseases, Free Radic. Biol. Med. 53 (2012) 314–327.
[32] T.V. Kudryashova, D.A. Goncharov, A. Pena, N. Kelly, R. Vanderpool, J. Baust, et al., HIPPO-integrin-linked kinase cross-talk controls self-sustaining proliferation and survival in pulmonary hypertension, Am. J. Respir. Crit. Care Med. 194 (2016) 866–877.
[33] D. Jia, Q. Zhu, H. Liu, C. Zuo, Y. He, G. Chen, A. Lu, Osteoprotegerin disruption attenuates HySu-induced pulmonary hypertension through integrin αvβ3/FAK/AKT pathway suppression, Circ. Cardiovasc. Genet. 10 (2017) e001591.
[34] Y. Zhang, K.J. Fan, Q. Sun, A.Z. Chen, W.L. Shen, Z.H. Zhao, et al., Functional screening for miRNAs targeting Smad4 identified miR-199a as a negative regulator of TGF-β signalling pathway, Nucleic Acids Res. 40 (2012) 9286–9297.
[35] K. Morita, K. Shirabe, A. Taketomi, Y. Soejima, T. Yoshizumi, H. Uchiyama, et al., Relevance of microRNA-18a and microRNA-199a-5p to hepatocellular carcinoma recurrence after living donor liver transplantation, Liver Transpl. 22 (2016) 665–676.
[36] C.Y. Hsu, T.H. Hsieh, C.F. Tsai, H.P. Tsai, H.S. Chen, Y. Chang, et al., miRNA-199a-5p regulates VEGFA in endometrial mesenchymal stem cells and contributes to the pathogenesis of endometriosis, J. Pathol. 232 (2014) 330–343.
[37] C.J. Holliday, R.F. Ankeny, H. Jo, R.M. Nerem, Discovery of shear- and side-specific mRNAs and miRNAs in human aortic valvular endothelial cells, Am. J. Physiol. Heart Circ. Physiol. 301 (2011) H856–H867.
[38] The Cancer Genome Atlas Research Network, The molecular taxonomy of primary prostate Cancer, Cell 163 (2015) 1011–1025.
[39] S. Mizuno, H.J. Bogaard, J. Gomez-Arroyo, A. Alhussaini, D. Kraskauskas, C.D. Cool, N.F. Voelkel, MicroRNA-199a-5p is associated with hypoxia-inducible factor-1alpha expression in lungs from patients with COPD, Chest 142 (2012) 663–672.
[40] S. Rane, M. He, D. Sayed, H. Vashistha, A. Malhotra, J. Sadoshima, et al., Downregulation of miR-199a derepresses hypoxia-inducible factor-1alpha and Sirtuin 1 and recapitulates hypoxia preconditioning in cardiac myocytes, Circ. Res. 104 (2009) 879–886.
[41] I. Hromadnikova, K. Kotlabova, L. Hympanova, L. Krofta, Gestational hypertension, preeclampsia and intrauterine growth restriction induce dysregulation of cardiovascular and cerebrovascular disease associated microRNAs in maternal whole peripheral blood, Thromb. Res. 137 (2016) 126–140.
[42] J. Lin, R.M. Zeng, R.N. Li, W.H. Cao, Aberrant DNA methylation of the P16, MGMT, and hMLH1 genes in combination with the methylenetetrahydrofolate reductase C677T genetic polymorphism and folate intake in gastric cancer, Genet. Mol. Res. 13 (2014) 2060–2068.
[43] R.M. Kok, D.E. Smith, R. Barto, A.M. Spijkerman, T. Teerlink, H.J. Gellekink, et al., Global DNA methylation measured by liquid chromatography-tandem mass spectrometry: analytical technique, reference values and determinants in healthy subjects, Clin. Chem. Lab. Med. 45 (2007) 903–911.
[44] B. Song, J. Ai, X. Kong, D. Liu, J. Li, Aberrant DNA methylation of P16, MGMT, and hMLH1 genes in combination with MTHFR C677T genetic polymorphism in gastric cancer, Pak. J. Med. Sci. 29 (2013) 1338–1343.
[45] G.H. Kim, J.J. Ryan, G. Marsboom, S.L. Archer, Epigenetic mechanisms of pulmonary hypertension, Pulm. Circ. 1 (2011) 347–356.
[46] J. Wang, L. Gong, Y. Tan, R. Hui, Y. Wang, Hypertensive epigenetics: from DNA methylation to microRNAs, J. Hum. Hypertens. 29 (2015) 575–582.
[47] T. Hassan, T.P. Carroll, P.G. Buckley, R. Cummins, S.J. O'Neill, N.G. McElvaney, C.M. Greene, miR-199a-5p silencing regulates the unfolded protein response in chronic obstructive pulmonary disease and α1-antitrypsin deficiency, Am. J. Respir. Crit. Care Med. 189 (2014) 263–273.